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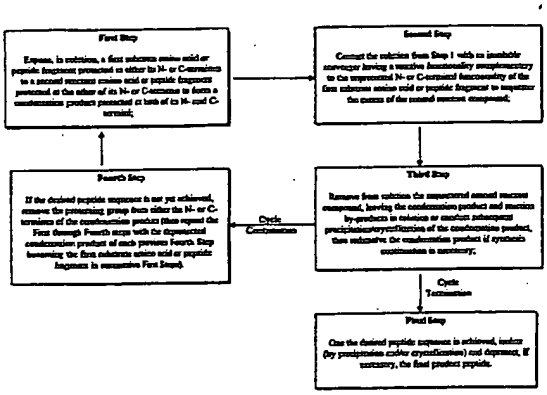
(54) Title: MINIMAL ISOLATION PEPTIDE SYNTHESIS PROCESS USING ION-EXCHANGE RESINS AS SCAVENGING AGENTS



(57) Abstract: A process for the production of a polypeptide having a pre-determined number and sequence of amino acid residues, comprising the steps of first exposing a first substrate amino acid or peptide fragment to a stoichiometric excess of a second reactant amino acid or peptide fragment to form a condensation product; second, contacting the reaction solution from the first step with an insoluble scavenger to sequester the excess of the second reactant amino acid or peptide fragment; third, removing from the solution the sequestered excess second reactant amino acid or peptide fragment; fourth, subjecting the reaction solution to a reaction which removes the protecting group from either the N- or C-terminus of the condensation product of the first step; and fifth, if necessary, repeating the first through fourth steps. The method is capable of large-scale production of peptides in solution, is not subject to the one-terminus-only limitation of the solid-phase method, possesses the "cleanliness" of the solid-phase method and, like the solid-phase method, is capable of automation. Most importantly, however, the method of the present invention does not require the frequent isolation of intermediates in a lengthy synthetic sequence nor, necessarily, the removal of all contaminating by-products from the reaction mixture prior to subsequent processing steps.

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capable of being automated and are well suited to the preparation of milligram to multi-gram quantities of peptides. A number of automated solid-phase peptide synthesizers are commercially available which employ a microcomputer to open and close valves which control the sequence and duration of flow of various reagents and washing solvents delivered to a resin on which the growing peptide is supported. When quantities of peptides exceeding hundreds of grams are required, the solid-phase synthetic method is generally inadequate, and resort is made to the solution-phase method.

In the solution-phase method, polypeptides are pieced together by classical solution chemistry which facilitates joining together individual amino acids, or di-, tri-, tetra- or oligopeptide fragments of the final polypeptide in which sites of unwanted reaction have been appropriately protected. The smaller fragments are themselves similarly prepared by piecing together individual amino acids or smaller protected fragments, etc. By the judicious mapping of such a synthesis, it is possible to minimize the number of steps required for the production of a desired end-product. Unlike the solid-phase method, the solution-phase method is free of the one-terminus-only limitation on the synthesis of a peptide. In the solution-phase method, a fragment formed in a previous step by reaction of an unprotected N-terminal amino function can be reacted in a subsequent step at its unprotected C-terminal carboxyl function. This possibility is not open to the solid-phase method since, as stated above, the growing peptide is "blocked" at C-terminus by attachment to the supporting resin.

Solution-phase peptide synthesis, despite being free of the one-terminus-only synthesis limitation of solid-phase peptide synthesis, suffers from a shortcoming: the need to frequently isolate and purify the growing peptide. All reactions are carried out in solution, resulting in a mixture which contains the desired product as well as unwanted unreacted reagents and by-products. After several steps of the synthesis, the solution would become burdened with appreciable amounts of these contaminants which, if left in the solution, would affect subsequent steps of the synthesis or burden the isolation and purification of the desired end-product peptide. As a consequence, it is prudent and often necessary to isolate intermediate products as the peptide synthesis goes forward. Each such isolation adds to the cost and time of the synthesis and lowers the overall yield of the desired peptide.

Methods of preparing small amounts of large libraries of compounds have become available with the advent in recent years of combinatorial chemistry techniques. Recently, the use of resins to sequester excess reagents and by-products from combinatorial chemistry steps has been described by D. L. Flynn, *et al.*, J. Am. Chem. Soc., **119**: 4874-4881 (1997). In the method described there, the authors use so-called CMR/R, or complementary molecular

possesses the "cleanliness" of the solid-phase method and, like the solid- phase method, is capable of automation. Most importantly, the method of the present invention does not require the frequent isolation of intermediates in a lengthy synthetic sequence nor, necessarily, the removal of all contaminants from the reaction mixture prior to subsequent processing steps.

In accordance with the principal embodiment of the present invention, there is provided a process for the synthesis of a polypeptide having a pre-determined number and sequence of amino acid residues. In its most general aspect, the process comprises sequentially the steps of first exposing, in solution, a first substrate amino acid or peptide fragment of the desired polypeptide product, the first substrate amino acid or peptide fragment being protected at either its N- or C-terminus, to a stoichiometric excess of a second reactant amino acid or peptide fragment of the desired polypeptide, the second reactant amino acid or peptide fragment being protected at the other of its N- or C-terminus, to form a condensation product of the substrate and reactant. The resulting condensation product is protected at both its N- and C-termini. In the second step of the process, the reaction solution is contacted with an insoluble scavenger having a reactive functionality complementary to the unprotected N- or C-terminal functionality of the first amino acid or peptide fragment, to sequester the excess of the second reactant amino acid or peptide fragment.

In the third step, the sequestered excess second reactant amino acid or peptide fragment is removed from the reaction solution, leaving the condensation product and reaction by-products in solution. Should it become necessary or desirable to decrease the volume of the reaction solution, which increases during the process of the present invention, the condensation product may be precipitated or crystallized, then place back in a solution of lesser volume. This solution, in the fourth step, is subjected to a reaction which removes the protecting group from either the N- or C-terminus of the condensation product of the first step. If, at this point, the desired polypeptide sequence is not yet achieved, the first through fourth steps are repeated as a cycle, with the deprotected condensation product of each previous fourth step becoming the substrate peptide fragment of each successive first step, until the desired peptide is produced. At the point at which the desired polypeptide sequence has been produced, the product is isolated and deprotected, if needed, of any terminal or side-chain protecting groups.

Description of the Drawing Figures

In the Drawing, which forms a part of the disclosure of the present invention:

FIGURE 1 is a schematic representation of the process steps for preparing polypeptides according to the method of the present invention.

(-OSu).

The protecting group for the N-terminus of the reactant or substrate amino acid or peptide fragment is a group which is "orthogonal" to protecting groups which are employed in protecting side-chain amino, hydroxyl, and carboxyl groups in either the reactant or substrate. Moreover, the N-terminal protecting group on the reactant or substrate amino acid or peptide fragment should be one which is easily removed under conditions which do not remove the side-chain protecting groups or the C-terminal blocking group of the other of the reactant or substrate. Two protecting groups are said to be "orthogonal" if chemical processes which are employed to remove one do not remove the other. A preferred N-terminal protecting group for the reactant or substrate amino acid or peptide fragment is one which is easily cleaved under conditions of hydrogenolysis, or catalytic hydrogenation. As sulfur is known to "poison" or inactivate hydrogenation catalysts, the preferred embodiment of the process of the present invention is limited to the synthesis of peptides which do not contain side-chain sulfhydryl or thioether groups; i.e., to the synthesis of non-cysteine-containing and non-methionine-containing peptides. The side-chain amino, hydroxyl and carboxyl blocking groups are selected from blocking groups well known in the art which are not cleaved under hydrogenolysis conditions. A preferred N-terminal amino protecting group for the reactant or substrate amino acids and peptide fragments utilized in the process of the present invention is the benzyloxycarbonyl group, sometimes termed the carbobenzoxy group, and denoted "Cbz" or simply "Z" in chemical shorthand. The Z group is easily cleaved by hydrogenolysis under mild conditions from the N-terminal amino groups of protected amino acids or peptides while leaving unaffected the less reactive protecting groups which have been used to protect side-chain functional groups. Preferred blocking groups for the C-terminus of the reactant or substrate amino acid or peptide fragment are simple ester groups such as the *tert*-butyl ester group and the like.

The substrate (unprotected at either its N-terminus or C-terminus) is allowed to react with the reactant (unprotected at the other of its N- or C-terminus) until analysis of aliquot samples periodically taken from the reaction mixture indicate substantially complete reaction. In the process of the present invention, the reactant is employed in stoichiometric excess to the amount of substrate in order to drive the condensation reaction to completion. Preferably, an amount greater than 1.0, up to about 1.1 moles of reactant amino acid or peptide fragment is employed per mole of substrate amino acid or peptide fragment. The amount of excess required in each particular coupling reaction will vary according to the chemical nature of the substrate and reactant to be coupled. However, it is within the skill of the process chemist to determine with a small-scale bench reaction the required molar ratio of reactant to substrate prior to committing to the cost of a large-scale preparation.

polymers manufactured by DEGUSSA AG, Weissfrauenstrasse 9, Frankfort am Main, Germany), and palladium supported on carbon, alumina (Al_2O_3), or silica (SiO_2). The hydrogenolysis leaves in the solution, once the catalyst has been removed by filtration, only the N-hydroxysuccinimide by-product of the initial coupling reaction, the N-terminally deprotected condensation product, and toluene which, together with carbon dioxide is the by-product of the Z-group hydrogenolysis. (It should be noted that the N-hydroxysuccinimide and toluene by-products interfere with neither the catalytic hydrogenation deprotection step, nor subsequent condensations. The absence of any need for their removal in multi-step syntheses is a particular advantage of the preferred process of the present invention.)

If, following removal of excess reactant amino acid or peptide fragment, the desired end-product polypeptide sequence has been completely constructed, the process is over, except for isolation of the polypeptide and deprotection by conventional methods, if needed or desired. The isolation is further simplified in the preferred embodiment of the process of the present invention, since the only contaminants remaining in the reaction solution are N-hydroxysuccinimide, tertiary amine salt (if the substrate or reactant amino acid or peptide fragment is employed in salt form, requiring neutralization prior to coupling), and toluene, all of which are easily removed by simple precipitation or crystallization of the desired end-product polypeptide. Those skilled in the art will realize that, when a polypeptide bearing, for example, a free N-terminus is the desired end-product or when recrystallization alone can serve to remove excess reactant amino acid or peptide fragment, isolation may be carried our following completion of a particular fourth or first step, respectively.

If, however, as is the more general case, the desired end-product polypeptide synthesis in incomplete following removal of excess reactant amino acid or peptide fragment, the deprotection coupling and scavenging steps are repeated using the deprotected condensation product of each cycle as the substrate material of each subsequent cycle until the desired polypeptide sequence is achieved.

Referring to Figure 2, one embodiment of a semi-automatic commercial scale processing system is shown for use in carrying out the process of the present invention. The system **100** comprises a first reactor vessel **102** for carrying out amino acid/peptide fragment condensation or coupling reactions, a second reactor **106** for carrying out de-protecting reactions, a resin column **104**, a filter **108**, a product holding tank **110**, first and second solvent tanks, **112** and **114**, and a numeric processor control unit **116**. While stainless steel reaction vessels may be used, it is preferred that all reactor vessels and tanks be glass-lined. It should be noted that the process layout in Figure 2 is schematic only. Representation of the filter as a plate-and-frame filter press

reactor vessel **102** and resin column **104**. The sensor is sensitive to excess reactant and is attached by feed-back control loop to numeric processor **116** and the signal from the recirculated product solution is used to control when the recirculation of the reaction solution through the resin column **104** may be stopped.

When analysis indicates that the reaction solution is substantially free of excess reactant, the numeric controller closes appropriate valves and opens others to transfer, by means of pump **122**, the eluate from resin column **102** to holding tank **114**. A first wash solvent from first solvent tank **112** is then circulated through resin column **104** and back to first solvent tank **112** to flush any remaining product from the column. This recirculating flush of column **104** may be for either a predetermined number of cycles or for a predetermined time, as controlled by numeric processor **116**. When the wash or flush of column **104** with the first solvent is complete, the first solvent wash solution, containing any product flushed from resin column **104**, is added to the contents of holding tank **114**.

Following the flush of resin column **104** by the first solvent, a second wash or flush of column **104** may be carried out if desired by employing a second solvent, initially held in second solvent tank **110**. As in the case of the wash of column **104** with the first solvent, the second solvent is recirculated between the column **104** and tank **110**, either for a pre-determined number of cycles, or for a pre-determined time, as controlled by numeric processor unit **116**. After complete flushing or washing of column **104**, the second solvent solution, containing any additional product washed from column **104** is added to the contents of holding tank **114**.

While the embodiment of the apparatus shown in Figure 2 depicts only two solvent wash tanks, **110** and **112**, it will be understood by those skilled in the chemical processing art that a third and subsequent tanks for additional solvents can be added as required. However, for most purposes, two tanks will suffice, with judicious choice of the wash solvents for each particular reaction step. Polar solvent can be employed as the wash solvent(s), with the limitation that the solvent must not be reactive with C-terminal activating groups, functional groups, or protecting groups on either the substrate or reactant. In addition, such solvents must serve to effectively solubilize both the substrate and the reactant. Suitable solvents include dimethylsulfoxide, dimethylformamide (DMF), N-methylpyrrolidone, and low molecular weight alcohols such as *iso*-propanol and the like, with cost and availability being concerns secondary to polarity and non-reactivity. Preferred solvents for the process of the present invention include dimethylformamide and *iso*-propanol.

At this step of the process, the holding tank **114** contains a solution comprising the coupled reaction product, together with the reaction solvent and the wash solvents, and (in the

catalyst and, if construction of the desired peptide is incomplete, transferred to first reactor vessel **102** for addition of next amino acid or peptide fragment to the growing peptide chain.

Process step 13: If construction of the desired peptide is incomplete, repeat steps 1-12 as required.

Process step 14: If construction of the peptide is complete, the contents of holding tank **114** from step 7 are collected for further processing.

The following examples are presented to enable one skilled in the art to better appreciate the process of the present invention. However, these examples are not to be read as limiting the scope of the invention as it is defined by the appended claims. Individual step numbers are designated in accordance with Figure 1. Numbering for cyclically repeated steps follows the format: step 1a, 2a, 3a and 4a for the first cycle of the process of the invention; step 1b, 2b, 3b and 4b for the second cycle; etc.

Example 1

Preparation of Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Alanyl-Phenylalanyl-Valyl-Lysyl(*tert*-butyloxycarbonyl)-Isoleucyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester

Step 1a) Preparation of Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester

Lys(Boc)-OMe.HCl (2.96 g, 10 mmol), and Z-Lys(Boc)-OSu (5.25 g, 11 mmol) are mixed with 40 mL (41.32 g) of *N*-Methyl-2-pyrrolidinone (NMP) in a 50 mL glass reactor, and the mixture is stirred at room temperature until the solids are dissolved. Diisopropylethylamine (DIEA) (1.24 g, 10.50 mmol) is added slowly to the reactor contents over a period of from about fifteen to thirty minutes. The resulting mixture is allowed to react at room temperature for about two hours. After this period of time, an aliquot sample of the reaction mixture is taken for analysis of the completeness of the reaction. If the analysis indicates that the reaction is incomplete, the reaction mixture is stirred at room temperature for an additional hour and the reaction mixture is analyzed again. When this analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2a-3a) Scavenging/Sequestration of Excess Reactant Z-Lys(Boc)-OSu

hydrogenation and reaction mixture analysis are repeated until the analysis indicates substantial completion of the hydrogenolysis reaction.

Upon completion of the hydrogenolysis reaction, the hydrogen is vented from the hydrogenator vessel, the vessel is purged with nitrogen, and the vessel contents are filtered to remove the catalyst. The hydrogenator vessel is rinsed with NMP, and the rinse solution is added to the reaction mixture filtrate.

Step 1b) Preparation of Benzyloxycarbonyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester

Z-Leu-OSu (3.98 g, 11 mmol), DIEA (1.29 g, 11 mmol) and the NMP/*iso*-propanol solution of the N-terminal deprotected dipeptide product of Step 4a (10 mmol, assuming complete reaction) are placed in a 1-liter glass reactor, and the mixture is stirred at room temperature until all solids are dissolved. The mixture is then stirred and allowed to react at room temperature for about two hours. At the end of this time, an aliquot sample of the reaction mixture is taken for analysis of the completeness of reaction. If the analysis indicates that the reaction is incomplete, the reaction mixture is stirred at room temperature for an additional hour and the reaction mixture is analyzed again. When the analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2b-3b) Scavenging/Sequestration of Excess Reactant Z-Leu-OSu

A column of aminomethyl resin (2 g) is prepared as described above in Step 2a-3a, and the NMP/*iso*-propanol reaction mixture of the blocked tripeptide from Step 1b is circulated repetitively through the resin column for about one hour, periodically analyzing the column eluate for absence of excess Z-Leu-OSu. When the analysis indicates substantially pure product, the NMP/*iso*-propanol reaction solution of the blocked tripeptide is set aside, and the column is washed by recirculating 15 mL (11.75 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked tripeptide product washed from the column, is set aside. The column is next washed by recirculating 30 mL (30.99 g) of NMP through the column for about thirty minutes. The NMP solution, containing blocked tripeptide product washed from the column, is set aside. The NMP/*iso*-propanol reaction solution of blocked tripeptide product and the *iso*-propanol and NMP wash solutions are combined.

Step 4b) N-Terminal Deprotection of the Benzyloxycarbonyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester Product of Step 1b

Using the same process as that of Step 4a, but without the addition of pTSA, the benzyloxycarbonyl protecting group on the Z-Ile-Leu-Lys(Boc)-Lys(Boc)-OMe tetrapeptide product of Step 1c is removed by hydrogenolysis.

Step 1d) Preparation of Benzyloxycarbonyl-Lysine(*tert*-butyloxycarbonyl)-Isoleucyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester

Z-Lys(Boc)-OSu (5.25 g, 11 mmol) is mixed in a 1-liter glass reactor vessel with the NMP/*iso*-propanol solution of the N-terminal deprotected tetrapeptide product from Step 4c. The mixture is stirred at room temperature until all solids are dissolved. The resulting mixture is allowed to react at room temperature for at least 2 hours, at which time an aliquot sample of the reaction mixture is analyzed for completeness of reaction. If the analysis indicates that the reaction is incomplete, the reaction mixture is stirred at room temperature for an additional hour and the reaction mixture is analyzed again. When analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2d-3d) Scavenging/Sequestration of Excess Reactant Z-Lys(Boc)-OSu and Precipitation/Crystallization of Condensation Product

A column of aminomethyl resin (2 gm) is prepared as described above in Step 2a-3a, and the NMP/*iso*-propanol reaction mixture of the blocked pentapeptide from Step 1d is then circulated repetitively through the resin column for about one hour in order to remove excess Z-Lys(Boc)-OSu. Circulation is continued, if necessary, until analysis indicates complete removal of the excess reactant. At this point, the NMP/*iso*-propanol reaction solution containing the blocked pentapeptide product is set aside. The resin column is then washed by recirculating 15 mL (11.75 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked pentapeptide product washed from the column, is set aside. The column is next washed by recirculating 30 mL (30.99 g) of NMP through the column for about thirty minutes. The NMP solution, containing blocked pentapeptide product washed from the column, is set aside. The resin column is given a final wash by recirculating 15 mL (11.75 g) of *iso*-propanol through the column for about thirty minutes. The NMP/*iso*-propanol reaction solution of blocked pentapeptide product, the NMP wash solution, and the two *iso*-propanol wash solutions are combined.

To the NMP/*iso*-propanol (± 350 mL) solution of the blocked pentapeptide is added precooled water (150 mL), causing precipitation/crystallization of the Z-Lys(Boc)-Ile-Leu-Lys(Boc)-Lys(Boc)-OMe pentapeptide product. The mixture is stirred for 24 hours, after which

continued, if necessary, until analysis indicates complete removal of the excess reactant. At this point, the NMP reaction solution containing the blocked hexapeptide product is set aside. The resin column is then washed by recirculating 15 mL (11.75 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked hexapeptide product washed from the column, is set aside. The column is next washed by recirculating 30 mL (30.99 g) of NMP through the column for about thirty minutes. The NMP solution, containing blocked hexapeptide product washed from the column, is set aside. The resin column is given a final wash by recirculating 15 mL (11.75 g) of *iso*-propanol through the column for about thirty minutes. The NMP reaction solution of blocked hexapeptide, the NMP wash solution, and the two *iso*-propanol wash solutions are combined.

Step 4e) N-Terminal Deprotection of the Benzyloxycarbonyl-Valyl-Lysyl(*tert*-butyloxycarbonyl)-Isoleucyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester Product of Step 1e

Using the same process as that of Step 4a, but without the addition of pTSA, the benzyloxycarbonyl protecting group on the Z-Val-Lys(Boc)-Ile-Leu-Lys(Boc)-Lys(Boc)-OMe hexapeptide product of Step 1e is removed by hydrogenolysis.

Step 1f) Preparation of Benzyloxycarbonyl-Phenylalanyl-Valyl-Lysine(*tert*-butyloxycarbonyl)-Isoleucyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester

Z-Phe-OSu (3.77 g, 9.50 mmol) is mixed in a 1-liter glass reactor vessel with the NMP/*iso*-propanol solution of the N-terminal deprotected hexapeptide product from Step 4e. The mixture is stirred at room temperature until all solids are dissolved. The resulting mixture is allowed to react at room temperature for at least 2 hours, at which time an aliquot sample of the reaction mixture is analyzed for completeness of reaction. If the analysis indicates that the reaction is incomplete, the reaction mixture is stirred at room temperature for an additional hour and the reaction mixture is analyzed again. When analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2f-3f) Scavenging/Sequestration of Excess Reactant Z-Phe-OSu

A column of aminomethyl resin (1.60 g) is prepared as described above in Step 2a-3a, and the NMP/iso-propanol reaction mixture of the blocked heptapeptide from Step 1f is then circulated repetitively through the resin column for about one hour in order to remove excess Z-Phe-OSu. Circulation is continued, if necessary, until analysis indicates complete removal of the excess reactant. At this point, the NMP/*iso*-propanol reaction solution containing the blocked

propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked octapeptide product washed from the column, is set aside. The column is next washed by recirculating 30 mL (30.99 g) of NMP through the column for about thirty minutes. The NMP solution, containing blocked octapeptide product washed from the column, is set aside. The resin column is given a final wash by recirculating 15 mL (11.75 g) of *iso*-propanol through the column for about thirty minutes. The NMP/*iso*-propanol reaction solution of blocked octapeptide, the NMP wash solution, and the two *iso*-propanol wash solutions are combined.

Step 4g N-Terminal Deprotection of the Benzyloxycarbonyl-Alanyl-Phenylalanyl-Valyl-Lysyl(*tert*-butyloxycarbonyl)-Isoleucyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester Product of Step 1g

Using the same process as that of Step 4a, but without the addition of pTSA, the benzyloxycarbonyl protecting group on the Z-Ala-Phe-Val-Lys(Boc)-Ile-Leu-Lys(Boc)-Lys(Boc)-OMe octapeptide product of Step 1g is removed by hydrogenolysis.

Step 1h) Preparation of Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl-Alanyl-Phenylalanyl-Valyl-Lysine(*tert*-butyloxycarbonyl)-Isoleucyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester

Z-Lys(Boc)-OSu (4.54 g, 9.50 mmol) is mixed in a 1-liter glass reactor vessel with the NMP/*iso*-propanol solution of the N-terminal deprotected octapeptide product from Step 4g. The mixture is stirred at room temperature until all solids are dissolved. The resulting mixture is allowed to react at room temperature for at least 2 hours, at which time an aliquot sample of the reaction mixture is analyzed for completeness of reaction. If the analysis indicates that the reaction is incomplete, the reaction mixture is stirred at room temperature for an additional hour and the reaction mixture is analyzed again. When this analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2h-3h) Scavenging/Sequestration of Excess Reactant Z-Lys(Boc)-OSu

A column of aminomethyl resin (1.60 g) is prepared as described above in Step 2a-3a, and the NMP/*iso*-propanol reaction mixture of the blocked nonapeptide from Step 1h is then circulated repetitively through the resin column for about one hour in order to remove excess Z-Lys(Boc)-OSu. Circulation is continued, if necessary, until analysis indicates complete removal of the excess reactant. At this point, the NMP/*iso*-propanol reaction solution containing the blocked nonapeptide product is set aside. The resin column is then washed by recirculating 15 mL (11.75 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution,

Aminomethyl resin (8.30 g) is mixed with 50 mL (47.10 g) of DMF in a second reactor vessel. The resulting mixture is stirred at room temperature until a homogeneous slurry is obtained. The resin/DMF slurry is charged to a glass column, the resin slurry is allowed to settle into a packed bed, and any excess DMF is drained from the column.

The DMF reaction mixture containing the Z-Phe-Gly-OMe product and excess Z-Phe-OSu reagent from Step 1a is then circulated repetitively through the resin column for about one hour in order to remove excess Z-Phe-OSu. Circulation is continued, if necessary, until analysis indicates complete removal of the excess reactant. At this point, the DMF reaction solution containing the blocked Z-Phe-Gly-OMe dipeptide product is set aside. The resin column is then washed by recirculating 20 mL (15.66 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked dipeptide product washed from the column, is set aside. The column is next washed by recirculating 40 mL (37.68 g) of DMF through the column for about thirty minutes. The DMF solution, containing blocked dipeptide product washed from the column, is set aside. The resin column is given a final wash by recirculating 20 mL (15.66 g) of *iso*-propanol through the column for about thirty minutes. The DMF reaction solution of blocked dipeptide, the DMF wash solution, and the two *iso*-propanol wash solutions are combined.

Step 4a) N-Terminal Deprotection of the Benzyloxycarbonyl-Phenylalanyl-Glycine-methyl Ester Product of Step 1a

Palladium-Deloxan® (0.90 g) and *para*-toluene sulfonic acid (pTSA) (10 g, 52.05 mmol) are placed in a 1-liter hydrogenator vessel. The hydrogenator vessel is flooded with argon, and the combined DMF/*iso*-propanol solutions from Step 2a-3a are charged to the hydrogenator vessel. The vessel is sealed and evacuated to a pressure of 20-25 inches of mercury (67.7 - 84.6 kPa) and purged three times with hydrogen. Hydrogen is then charged to the hydrogenator vessel to a pressure of 35-45 psi (234.5-310.3 kPa) and the mixture is stirred at about 30°C for about 2 hours.

The hydrogen is then vented from the hydrogenator vessel, and the vessel is purged twice with nitrogen. An aliquot sample of the reaction mixture is taken for analysis of the completeness of reaction. If the analysis indicates incomplete reaction, the hydrogenator vessel is purged twice with hydrogen, recharged with hydrogen to a pressure of 35-45 psi (234.5-310.3 kPa), and the mixture is again stirred at a temperature of about 30°C for an additional one hour. The hydrogenator vessel is evacuated and then purged twice with nitrogen, and an aliquot sample of the reaction mixture is again taken for analysis of the completeness of reaction. The above steps of hydrogenation and reaction mixture analysis are repeated until the analysis indicates substantial completion of the hydrogenolysis reaction.

Using the same process as that of Step 4a, but without the addition of pTSA, the benzyloxycarbonyl protecting group on the N-terminus of the Z-Lys(Boc)-Phe-Gly-OMe tripeptide product of Step 1b is removed by hydrogenolysis.

Step 1c) Preparation of Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Glycine-methyl Ester

Z-Lys(Boc)-OSu (26.20 g, 55 mmol) and the DMF/*iso*-propanol solution of the deprotected tripeptide product of Step 4b are placed in a 1-liter glass reactor, and the mixture is stirred at room temperature until all solids are dissolved. The mixture is then stirred and allowed to react at room temperature for about two hours. At the end of this time, an aliquot sample of the reaction mixture is taken for analysis of the completeness of reaction. If the analysis indicates that the reaction is incomplete, the mixture is stirred at room temperature for an additional hour and the reaction mixture is again analyzed. When this analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2c-3c) Scavenging/Sequestration of Excess Reactant Z-Lys(Boc)-OSu

A column of aminomethyl resin (8.30 g) is prepared as described above in Step 2a-3a, and the DMF/*iso*-propanol reaction mixture from Step 1c is circulated repetitively through the resin column for about one hour, periodically analyzing the column eluate for absence of excess Z-Lys(Boc)-OSu. When the analysis indicates substantially pure product, the DMF/*iso*-propanol solution of reaction product is set aside, and the column is washed by recirculating 20 mL (15.66 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked tetrapeptide product washed from the column, is set aside. The column is next washed by recirculating 40 mL (37.68 g) of DMF through the column for about thirty minutes. The DMF solution, containing blocked tetrapeptide product washed from the column, is set aside. The resin column is given a final wash by recirculating 20 mL (15.66 g) of *iso*-propanol through the column for about thirty minutes. The DMF/*iso*-propanol reaction solution of blocked tetrapeptide product and the *iso*-propanol and DMF wash solutions are combined.

Step 4c) N-Terminal Deprotection of the Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Glycine-methyl Ester Product of Step 1c

Using the same process as that of Step 4a, but without the addition of pTSA, the benzyloxycarbonyl protecting group on the N-terminus of the Z-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe tetrapeptide product of Step 1c is removed by hydrogenolysis.

product from Step 4d. The mixture is stirred at room temperature until all solids are dissolved. The resulting mixture is allowed to react at room temperature for at least 2 hours, at which time an aliquot sample of the reaction mixture is analyzed for completeness of reaction. If the analysis indicates that the reaction is incomplete, the mixture is stirred at room temperature for an additional hour and the reaction mixture is again analyzed. When this analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2e-3e) Scavenging/Sequestration of Excess Reactant Z-Lys(Boc)-OSu

A column of aminomethyl resin (3.45 g) is prepared as described above in Step 2a-3a, and the NMP reaction mixture of the blocked hexapeptide from Step 1e is then circulated repetitively through the resin column for about one hour in order to remove excess Z-Lys(Boc)-OSu. Circulation is continued, if necessary, until analysis indicates complete removal of the excess reactant. At this point, the NMP reaction solution containing the blocked hexapeptide product is set aside. The resin column is then washed by recirculating 20 mL (15.66 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked hexapeptide product washed from the column, is set aside. The column is next washed by recirculating 40 mL (41.32 g) of NMP through the column for about thirty minutes. The NMP solution, containing blocked hexapeptide product washed from the column, is set aside. The resin column is given a final wash by recirculating 20 mL (15.66 g) of *iso*-propanol through the column for about thirty minutes. The NMP reaction solution of blocked hexapeptide, the NMP wash solution, and the two *iso*-propanol wash solutions are combined.

Step 4e) N-Terminal Deprotection of the Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Alanyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Glycine-methyl Ester Product of Step 1e

Using the same process as that of Step 4a, but without the addition of pTSA, the benzyloxycarbonyl protecting group on the N-terminus of the Z-Lys(Boc)-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe hexapeptide product of Step 1e is removed by hydrogenolysis.

Step 1f) Preparation of Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Alanyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Glycine-methyl Ester

Z-Lys(Boc)-OSu (9.89 g, 20.72 mmol) and the NMP/*iso*-propanol solution of the deprotected hexapeptide product of Step 4e are placed in a 1-liter glass reactor, and the mixture is stirred at room temperature until all solids are dissolved. The resulting mixture allowed to react at

reaction is incomplete, the mixture is stirred at room temperature for an additional hour and the reaction mixture is again analyzed. When this analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2g-3g) Scavenging/Sequestration of Excess Reactant Z-Leu-OSu and Precipitation/Crystallization of the Condensation Product

A column of aminomethyl resin (3.45 g) is prepared as described above in Step 2a-3a, and the NMP/*iso*-propanol reaction mixture from Step 1g is circulated repetitively through the resin column for about one hour, periodically analyzing the column eluate for absence of excess Z-Leu-OSu. When the analysis indicates substantially pure product, the NMP/*iso*-propanol solution of reaction product is set aside, and the column is washed by recirculating 40 mL (31.32 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing blocked octapeptide product washed from the column, is set aside. The column is next washed by recirculating 80 mL (81.64 g) of NMP through the column for about thirty minutes. The NMP solution, containing blocked octapeptide product washed from the column, is set aside The resin column is given a final wash by recirculating 40 mL (31.32 g) of *iso*-propanol through the column for about thirty minutes. The NMP/*iso*-propanol reaction solution of blocked octapeptide product, and the *iso*-propanol and NMP wash solutions are combined.

To the NMP/*iso*-propanol (± 500 mL) solution of the blocked octapeptide is added precooled water (200 mL), causing precipitation/crystallization of the Z-Leu-Lys(Boc)-Lys(Boc)-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe octapeptide product. The octapeptide is isolated by filtration, rinsed with NMP/water (500/200 mL) and 2 liters of water, then dried under vacuum. The yield of Z-Leu-Lys(Boc)-Lys(Boc)-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe is 22.75 g (82% of theoretical yield from Z-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe).

Step 4g) N-Terminal Deprotection of the Benzyloxycarbonyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Alanyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Glycine-methyl Ester Product of Step 3g

Z-Leu-Lys(Boc)-Lys(Boc)-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe (19.18 g, 13.08 mmol) is mixed with 200 mL of NMP in a 1-liter glass reactor. Then, using the same process as that of Step 4a, but without the addition of pTSA, the benzyloxycarbonyl protecting group on the N-terminus of the blocked octapeptide product (of Step 3g) is removed by hydrogenolysis.

Step 1h) Preparation of Benzyloxycarbonyl-Phenylalanyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Alanyl-Lysyl(*tert*-

butyloxycarbonyl)-Alanyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Glycine-methyl Ester

Z-Lys(Boc)-OSu (6.55 g, 13.73 mmol) and the NMP/*iso*-propanol solution of the deprotected nonapeptide product of Step 4h are placed in a 1-liter glass reactor, and the mixture is stirred at room temperature until all solids are dissolved. The resulting mixture is allowed to react at room temperature for about two hours, at which time an aliquot sample of the reaction mixture is taken for analysis of the completeness of reaction. If the analysis indicates that the reaction is incomplete, the mixture is stirred at room temperature for an additional hour and the reaction mixture is again analyzed. When this analysis indicates the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring. (Note: Scavenging/sequestration of excess reactant Z-Lys(Boc)-OSu, as per a final Step 2i-3i sequence, was not conducted prior to isolation of the final condensation product in Example 2.)

Step 5) Isolation of Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Alanyl-Lysyl(*tert*-butyloxycarbonyl)-Lysyl(*tert*-butyloxycarbonyl)-Phenylalanyl-Glycine-methyl Ester

To the NMP/*iso*-propanol (± 260 mL) solution of the blocked decapeptide from Step 1i is added precooled water (150 mL), causing precipitation/crystallization of the Z-Lys(Boc)-Phe-Leu-Lys(Boc)-Lys(Boc)-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe decapeptide product. The decapeptide is isolated by filtration, rinsed with 1 liter of methanol, and dried under vacuum. The yield of Z-Lys(Boc)-Phe-Leu-Lys(Boc)-Lys(Boc)-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe is 19.02 g (79% of theoretical yield from Z-Leu-Lys(Boc)-Lys(Boc)-Ala-Lys(Boc)-Lys(Boc)-Phe-Gly-OMe, 41% overall yield).

Example 2

Preparation of 9-Fluorenylmethoxycarbonyl-*beta*-Alanyl-Leucyl-Alanyl-Leucine-*tert*-butyl Ester

Step 1) Preparation of Benzyloxycarbonyl-Alanyl-Leucine- *tert*-butyl Ester

Leu-O*t*Bu·HCl (184 g, 0.82 mol), and Z-Ala-OSu (290 g, 0.91 mol) are mixed with 1.9 liters (1790 g) of dimethylformamide in a 5-liter glass reactor, and the mixture is stirred at room temperature until the solids are dissolved. Diisopropylethylamine (115 g (0.97 mol) is added slowly to the reactor contents over a period of from about fifteen to thirty minutes. The resulting mixture is allowed to react at room temperature for about two hours. Aliquot samples of the reactor contents are then taken for analysis of the completeness of the coupling reaction. When

incomplete reaction, the hydrogenator vessel is purged twice with hydrogen, recharged with hydrogen to a pressure of 35-45 psi (234.5-310.3 kPa) and the mixture is again stirred at a temperature of about 30°C for an additional hour. The hydrogenator vessel is evacuated and then purged twice with nitrogen, and an aliquot sample of the reaction mixture is again taken for analysis of the completeness of reaction. The above steps of hydrogenation and reaction mixture analysis are repeated until the analysis indicates substantial completion of the hydrogenolysis reaction.

Upon completion of the hydrogenolysis reaction, the hydrogenator vessel is vented to vacuum, purged with nitrogen, and the vessel contents are filtered to remove the catalyst. The hydrogenator vessel is rinsed with dimethylformamide, and the rinse solution is added to the reaction mixture filtrate.

Step 1') - Preparation of Benzyloxycarbonyl-Leucyl-Alanyl-Leucine- *tert*-butyl Ester

Z-Leu-OSu (328 g, 0.90 mol) and the solution of the deprotected dipeptide product of step 4) (0.82 mol, assuming complete reaction) are placed in a 12-liter glass reactor, and the mixture is stirred at room temperature until all solids are dissolved. The mixture is then stirred at room temperature for about two hours. At the end of this time, an aliquot sample of the reaction mixture is taken for analysis of the completeness of the coupling reaction. If the analysis indicates that the reaction is incomplete, the mixture is stirred at room temperature for an additional hour and the reaction mixture is again analyzed. When these analyses indicate the coupling reaction is complete, the reactor contents are held at room temperature with slow stirring.

Steps 2'-3') - Scavenging/Sequestration of Excess Reactant Z-Leu-OSu

A column of aminomethyl resin (164 g) is prepared as described above in steps 2) and 3) and the reaction mixture from step 1') is circulated repetitively through the resin column for about one hour, periodically analyzing the column eluate for absence of excess Z-Leu-OSu. When the analysis indicates substantially pure tripeptide product, the solution of Z-Leu-Ala-Leu-O-*t*-Bu is set aside, and the column is washed by recirculating 785 mL (615 g) of *iso*-propanol through the column for about thirty minutes. The *iso*-propanol solution, containing Z-Leu-Ala-Leu-O-*t*-Bu tripeptide product washed from the column, is set aside. The column is next washed by recirculating 1.56 liters (1470 g) of dimethylformamide through the column for about thirty minutes. The DMF solution, containing of Z-Leu-Ala-Leu-O-*t*-Bu tripeptide product washed from the column is set aside. The resin column is given a final wash by circulating 785 mL (615 g) of *iso*-propanol through the column for about thirty minutes. The original solution of of Z-Leu-Ala-

At this point, the filter cake is washed with approximately 4 kg of acetonitrile, with the filtrate wash being collected in an appropriate receiver. The acetonitrile-wet filter cake is aspirated to dryness for about one hour, and then transferred to a glass drying tray and dried under vacuum and nitrogen flow at 30°C. The yield of Fmoc-β-Ala-Leu-Ala-Leu-O-*t*-Bu is 30.5 g (60% of theoretical yield).

2. The process according to Claim 1 wherein the protecting group removed in step d) is removed from the N-terminus.

3. The process according to Claim 1 wherein the protecting group removed in step d) is removed from the C-terminus.

4. The process according to Claim 1 wherein the insoluble scavenger employed in step b) is selected from amine- and carboxyl-functionalized resins.

5. The process according to Claim 2 wherein the insoluble scavenger employed in step b) is an amine-functionalized resin.

6. The process according to Claim 3 wherein the insoluble scavenger employed in step b) is a carboxyl-functionalized resin.

7. The process according to Claim 5 wherein said resin is an aminomethyl-functionalized polystyrene-divinylbenzene resin.

8. A process for the synthesis of a polypeptide having a pre-determined number and sequence of amino acid residues comprising the steps of:

a) exposing a solution of a substrate amino acid or fragment of said polypeptide, said substrate having a C-terminal protecting group not removable by hydrogenolysis, to a stoichiometric excess of a reactant amino acid or a fragment of said polypeptide, said reactant having an N-terminal protecting group removable by hydrogenolysis, to form a condensation product;

b) contacting the solution from step a) with an amine-functionalized resin to sequester the excess of said reactant amino acid or peptide fragment;

c) removing from the solution the sequestered excess of said reactant amino acid or peptide fragment leaving the condensation product and reaction

b) contacting the solution from step a) with an amine-functionalized resin to sequester and to remove from solution the excess of the reactant amino acid or peptide fragment leaving the condensation product and reaction by-products in solution or conducting subsequent precipitation or crystallization of the condensation product, then redissolving the condensation product if synthesis continuation is necessary or desired;

c) subjecting the solution from step b) to catalytic hydrogenation conditions to remove the benzyloxycarbonyl protecting group from the N-terminus of said condensation product;

d) removing, by filtration, the hydrogenation catalyst from the solution;

e) repeating steps a) through d) until the desired polypeptide has been produced; and

f) isolating and deprotecting, if needed or desired, the product polypeptide.

14. A process according to claim 13 wherein the polypeptide synthesized is Benzyloxycarbonyl-Lysyl(*tert*-butyloxycarbonyl)-Alanyl-Phenylalanyl-Valyl-Lysyl(*tert*-butyloxycarbonyl)-Isoleucyl-Leucyl-Lysyl(*tert*-butyloxycarbonyl)-Lysine(*tert*-butyloxycarbonyl)-methyl Ester.

15. A process according to claim 13 wherein the polypeptide synthesized is 9-Fluorenylmethoxycarbonyl-*beta*-Alanyl-Leucyl-Alanyl-Leucine-*tert*-butyl Ester.

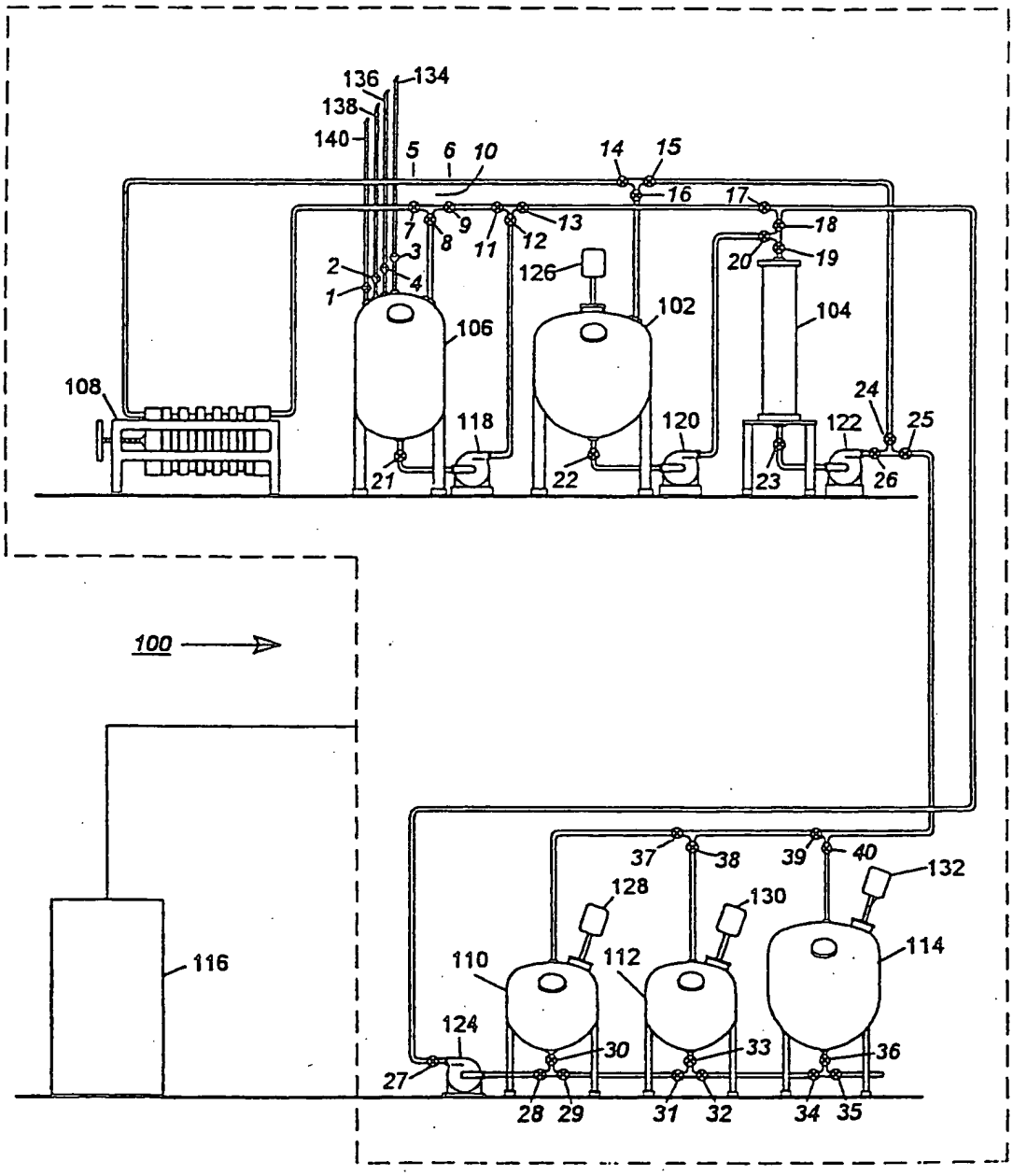
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Figure 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/14152

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	S D BROWN & R W ARMSTRONG : "Synthesis of tetrasubstituted ethylenes on solid support via resin capture " JOURNAL OF THE AMERICAN CHEMICAL SOCIETY., vol. 118, no. 26, 3 July 1996 (1996-07-03), pages 6331-6332, XP002149793 AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC., US ISSN: 0002-7863 cited in the application the whole document	1-15
A	L M GAYO & M J SUTO: "Ion-exchange resins for solution phase parallel synthesis of chemical libraries" TETRAHEDRON LETTERS., vol. 38, no. 4, 27 January 1997 (1997-01-27), pages 513-516, XP002149794 ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM., NL ISSN: 0040-4020 cited in the application the whole document	1-15
A	WO 97 42230 A (WARNER-LAMBERT) 13 November 1997 (1997-11-13) the whole document	1-15
A	D L FLYNN ET AL.: "Chemical library purification strategy based on principles of complementary molecular reactivity and molecular recognition " JOURNAL OF THE AMERICAN CHEMICAL SOCIETY., vol. 119, no. 21, 28 May 1997 (1997-05-28), pages 4874-4881, XP002149798 AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC., US ISSN: 0002-7863 cited in the application the whole document	1-15